Micromonospora schwarzwaldensis sp. nov., a producer of telomycin, isolated from soil

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A Gram-stain-positive, spore-forming actinomycete strain (HKI0641^T) was isolated from a soil sample collected in the Black Forest, Germany. During screening for antimicrobial natural products this bacterium was identified as a producer of the antibiotic telomycin. Morphological characteristics and chemotaxonomic data indicated that the strain belonged to the genus *Micromonospora*. The peptidoglycan of strain HKI0641^T contained *meso*-diaminopimelic acid, and the fatty acid profile consisted predominantly of anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and $C_{16:0}$. MK-10(H_4), MK-10(H_2) and MK-10 were identified as the major menaquinones. To determine the taxonomic positioning of strain HKI0641^T, we computed a binary tanglegram of two rooted phylogenetic trees that were based upon 16S rRNA and *gyrB* gene sequences. The comparative analysis of the two common classification methods strongly supported the phylogenetic affiliation with the genus *Micromonospora*, but it also revealed discrepancies in the assignment at the level of the genomic species. 16S rRNA gene sequence analysis identified *Micromonospora coxensis* DSM 45161^T (99.1 % sequence similarity) and *Micromonospora marina* DSM 45555^T (99.0 %) as the nearest taxonomic neighbours, whereas the *gyrB* sequence of strain HKI0641^T indicated a closer relationship to *Micromonospora aurantiaca* DSM 43813^T (95.1 %). By means of DNA–DNA hybridization experiments, it was possible to resolve this issue and to clearly differentiate strain HKI0641^T from other species of the genus *Micromonospora*. The type strains of the aforementioned species of the genus *Micromonospora* could be further distinguished from strain HKI0641^T by several phenotypic properties, such as colony colour, NaCl tolerance and the utilization of carbon sources. The isolate was therefore assigned to a novel species of the genus *Micromonospora*, for which the name *Micromonospora schwarzwaldensis* sp. nov. is proposed. The type strain is HKI0641^T (=DSM 45708^T=CIP 110415^T).

Micromonospora is the type genus of the family *Micromonosporaceae* Krasil'nikov 1938, emend. Zhi, Li and Stackebrandt 2009 within the suborder *Micromonosporineae* in the order *Actinomycetales* (Genilloud, 2012; Stackebrandt *et al.*, 1997; Zhi *et al.*, 2009). This family contains several genera which are morphologically distinct, but chemotaxonomically similar (Goodfellow *et al.*, 1990). At the time of writing, the 'List of Prokaryotic Names with Standing in Nomenclature' includes 50 species and seven subspecies in the genus *Micromonospora* (Euzéby, 2012). The majority of these species have been isolated from soil, freshwater or marine habitats (Carro *et al.*, 2012; Genilloud, 2012; Luedemann *et al.*, 1963). Like other actinomycetes, species of the genus *Micromonospora* are best known for synthesizing bioactive secondary metabolites, especially aminoglycoside, enediyne and oligosaccharide antibiotics (Bérdy, 2005). Their metabolic proficiency was confirmed in whole-genome sequencing projects, which showed that these organisms

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Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA and *gyrB* gene sequences of strain HKI0641^T are KC517406 and KC517407, respectively.

Five supplementary tables and four supplementary figures are available with the online version of this paper.

dedicate a large portion of their genetic capacity to the biosynthesis of natural products (Nett *et al.*, 2009; Alonso-Vega *et al.*, 2012). While the biological function of most of these molecules still remains elusive, there is mounting evidence that some of them contribute to plant health, e.g. by alleviating metal-induced oxidative stress (Dimkpa *et al.*, 2009) or by suppressing the growth of phytopathogens (Raaijmakers & Mazzola, 2012). The importance of species of the genus *Micromonospora* for soil ecology, including plant growth and development, has recently been recognized (Hirsch & Valdés, 2010). During a survey of potential biocontrol agents from soil in the Black Forest (Schwarzwald), 48 strains were isolated. Extracts of strain HKI0641^T showed strong activities in the agar diffusion assay against various Gram-reaction-positive bacteria as well as fungi, suggesting the production of antimicrobial natural products. Here, we report a comprehensive phenotypic and phylogenetic characterization of strain HKI0641^T.

The soil samples were collected in the Black Forest near Forbach, Germany, in 2000. Strain HKI0641^T was isolated from the flooding zone of the Schwarzenbach dam. The corresponding sample (pH 5.5) contained significant amounts of loam, but also some plant debris. To promote the isolation of spore-forming actinomycetes, all samples were initially dried and heated for 1 h at 80 °C. Afterwards, 1 g of each sample was suspended in 10 ml 0.85 % NaCl (w/v) and mixed on a shaker for 30 min. After sedimentation of the soil particles the supernatants were diluted to 10^{-4}, 10^{-5} and 10^{-6} with 0.85 % NaCl (w/v). Aliquots of these suspensions were spread over plates containing humic acid-vitamin agar (Hayakawa & Nonomura, 1987) supplemented with nalidixic acid (20 μg ml^{-1}) and cycloheximide (30 μg ml^{-1}). The plates were incubated at 28 °C for three weeks. All isolates were purified and maintained on yeast extract–malt extract (ISP-2) agar (Shirling & Gottlieb, 1966). Pure cultures were preserved at −80 °C as a mixture of hyphae and few spores in liquid ISP-2 medium and glycerol medium [8.8 % glycerol, 0.18 % $(NH_4)_2SO_4$, 0.09 % Na-citrate, 1.26 % K_2HPO_4, 0.36 % H_2PO_4 and 0.01 % $MgSO_4$]. Stock cultures in liquid ISP-2 medium supplemented with 5 % DMSO were additionally maintained in the vapour phase of liquid nitrogen. To identify potential producers of bioactive metabolites, the supernatants of isolated strains, grown in liquid ISP-2 medium for 7 days, were subjected to an agar diffusion assay as previously described (Nett & Hertweck, 2011). Based upon the antimicrobial effects in this primary screening, strain HKI0641^T was selected for further taxonomic analysis.

Genomic DNA as template for PCR was extracted using the DNeasy Blood and Tissue kit (Qiagen). PCR amplification of the 16S rRNA gene was performed using primers Fw-16S (5′-GTCTCTGGGCCGATACTGACGC-3′) and Rev-16S (5′-CGGCTACCTTGTTACGAC TTCGTC-3′). The sequencing of the *gyrB* gene was performed as described by Garcia *et al.* (2010). 16S rRNA gene and *gyrB* sequences of strain HKI0641^T served as probes to search for similar sequences using the BLASTN module of EPoS (Griebel *et al.*, 2008). Representative sequences were manually selected (Table S1 available in IJSEM Online) and aligned with the CLUSTAL W module of EPoS using default parameters. This approach resulted in a multiple alignment of 1413 and 1032 sites after removing all gap columns. The alignments were subsequently used to compute sequence similarities using the R-package APE (Paradis *et al.*, 2004) as well as the phylogenetic trees employing the neighbour-joining (NJ) module of EPoS based on the Kimura model. The NJ calculation was performed utilizing *Actinoplanes regularis* DSM 43151^T as the outgroup and 500 bootstrap replicates to assess the stability of the grouping.

The almost complete 16S rRNA gene sequence of strain HKI0641^T was a continuous stretch of 1460 bp between positions 25 and 1518 of the *Escherichia coli* numbering (Brosius *et al.*, 1978). The complete signature nucleotide patterns associated with the order *Actinomycetales* and the family *Micromonosporaceae* were identified (Table S2; Zhi *et al.*, 2009). The sequence-based similarity calculations indicated that the closest relatives of strain HKI0641^T were *Micromonospora coxensis* DSM 45161^T (99.1 %) and *Micromonospora marina* DSM 45555^T (99.0 %). Due to the high levels of relatedness of strains of species of the genus *Micromonospora* based on their 16S rRNA gene sequences (Carro *et al.*, 2010; Koch *et al.*, 1996), we set out to verify the 16S rRNA gene-derived phylogenetic classification by applying a *gyrB*-based method (Kasai *et al.*, 2000). According to this analysis, however, strain HKI0641^T should be affiliated with *Micromonospora. aurantiaca* DSM 43813^T rather than with the aforementioned species. We illustrated the observed discrepancy by comparing both inferred trees in a tanglegram (Böcker *et al.*, 2009). This graphical juxtaposition showed several lines crossing, thereby indicating significant methodological bias (Fig. 1). To resolve the phylogenetic grouping of strain HKI0641^T, spectroscopic DNA–DNA hybridization experiments were performed in duplicate according to the methods of De Ley *et al.* (1970) and Huss *et al.* (1983). The required DNA was obtained following cell disruption and purification of the resulting crude lysate via column chromatography on hydroxyapatite (Cashion *et al.*, 1977). The highest DNA–DNA reassociation value was obtained between strain HKI0641^T and *M. aurantiaca* DSM 43813^T (mean value, 44.3 %), whereas the corresponding values with *M. marina* DSM 45555^T (34.9 %) and *M. coxensis* DSM 45161^T (11.5 %) were significantly lower (Table S3). This result corroborated the superiority of *gyrB* gene sequence analysis for inferring intrageneric relationships in the genus *Micromonospora*. Since the phylogenetic definition of a species generally excludes strains with <70 % DNA–DNA relatedness, it was evident that the isolate HKI0641^T represented a distinct species (Wayne *et al.*, 1987).

Subsequently, the phenotypic features of the novel strain were analysed. Gram staining and cell morphology were examined under a phase-contrast microscope using 24 h-old cultures grown on ISP-2 agar at 28 °C. For scanning

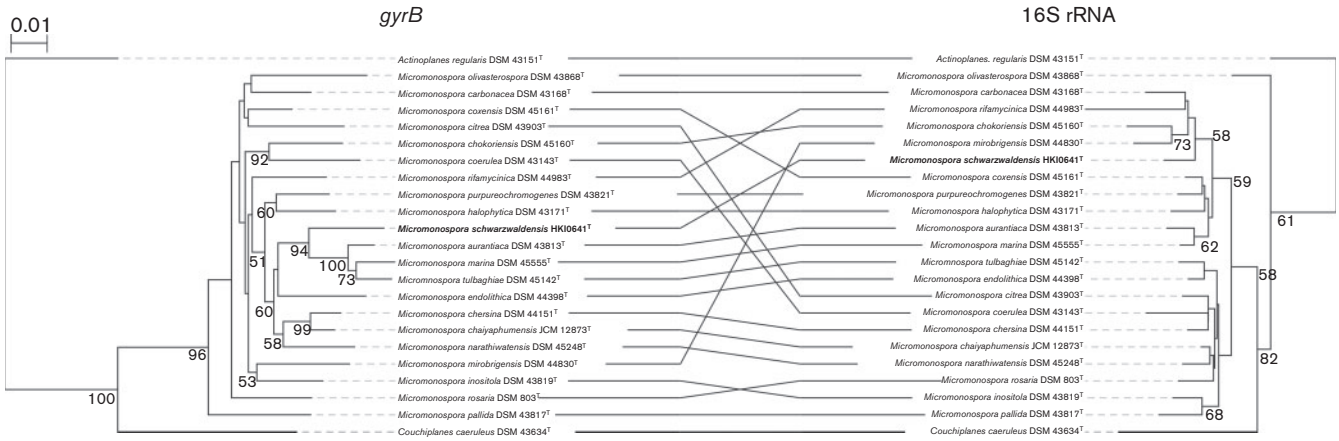

Fig. 1. Tanglegram comparing neighbour-joining phylogenetic trees based upon *gyrB* (left) and 16S rRNA (right) gene sequences from strain HKI0641^T and members of the family *Micromonosporaceae*. *Actinoplanes regularis* DSM 43151^T was used as an outgroup. The numbers on the branches indicate the bootstrap values (%) from 500 resamplings; only values >50 % are indicated. Bar, 0.01 substitutions per nucleotide position.

electron microscopy, a 28 day-old agar culture was suspended in a phosphate-buffered salt solution. The cells were fixed with 0.5 % glutaraldehyde, washed and dehydrated in ascending ethanol concentrations. Afterwards the samples were critical-point dried using liquid CO_2 and sputter coated with platinum using a SCD005 sputter coater (BAL-TEC) to avoid surface charging. Finally the specimens were investigated with a field emission scanning electron microscope (LEO-1530 Gemini; Carl Zeiss NTS). The fatty acid profile was determined according to the method described by Groth *et al.* (1996). For quinone and polar lipid analysis, cells were grown in ISP-2 medium at 28 °C. Quinone analysis was performed according to the procedure described by Collins *et al.* (1977). Polar lipids were determined according to the methods of Minnikin *et al.* (1979) and Collins & Jones (1980). Isomers of diaminopimelic acid in whole cells hydrolysates were analysed by TLC on cellulose (Schleifer & Kandler, 1972). The occurrence of mycolic acids was determined by TLC as described by Minnikin *et al.* (1975). Whole-cell sugars were examined according to the method of Schumann (2011). The utilization of carbon sources was investigated using the API 50 CH B system (bioMérieux). Temperature-dependent growth was analysed on ISP-2 agar at the following incubation temperatures: 4, 10, 20, 25, 28, 37 and 45 °C. Tolerance to NaCl and pH were determined on ISP-2 agar at 28 °C by the addition of 2, 4, 6, 8, 10 or 15 % (w/v) NaCl and using a pH range from 4 to 10. Susceptibility to antibiotics was tested on ISP-2 agar at 28 °C.

The morphological and chemical properties of strain HKI0641^T are consistent with its classification as a member of the genus *Micromonospora* (Genilloud, 2012). The isolate developed substrate hyphae on ISP-2 agar, oatmeal agar (ISP-3) and on inorganic salts–starch agar (ISP-4) (Shirling & Gottlieb, 1966). In comparison with the former three media, the growth on glycerol–asparagine agar (ISP-5) was delayed (Table S4). Abundant black spores were observed on ISP-2 agar (Fig. 2, Fig. S1), but no soluble pigments were observed in any of the media tested. Aerial mycelium was always absent. *M. aurantiaca* DSM 43813^T, *Micromonospora purpureochromogenes* DSM 43821^T and *Micromonospora tulbaghiae* DSM 45142^T exhibited the same growth profile as strain HKI0641^T, albeit they differed in sporulation and in the colour of their colonies. The growth temperature of strain HKI0641^T ranged from 20 to 37 °C, with optimal growth occurring at 28 °C. Except for *M. aurantiaca* DSM 43813^T, which did not grow below 25 °C, all other tested strains of species of the genus

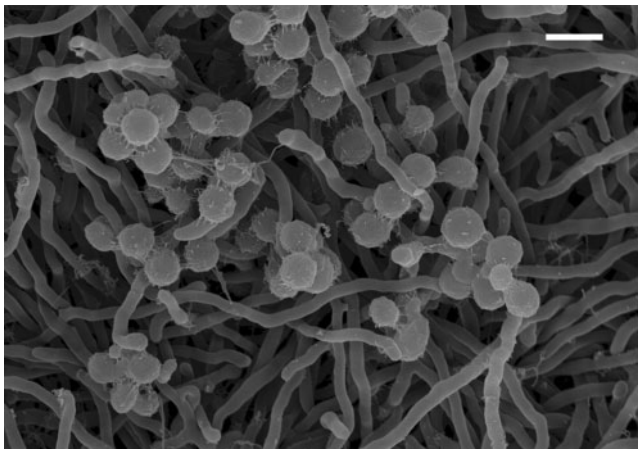

Fig. 2. Scanning electron micrograph of strain HKI0641^T cultivated at 28 °C on ISP-2 agar for 28 days. Bar, 1 μm.

Micromonospora thrived in the same temperature range. Most strains tolerated pH 6–10 and up to 2 % NaCl. Only *M. tulbaghiae* DSM 45142^T was restricted to pH 7–9. Strain HKI0641^T also grew at elevated NaCl concentrations up to 4 %. The fatty acid profile of strain HKI0641^T was dominated by branched-chain fatty acids in accordance with those of other species of the genus *Micromonospora*. However, some qualitative and quantitative differences were found. While anteiso-$C_{15:0}$ was a major constituent of strain HKI0641^T (19.9 %) and *M. aurantiaca* DSM 43813^T (16.4 %), the same fatty acid was much less notable in *M. marina* DSM 45555^T (4.9 %) and *M. purpureochromogenes* DSM 43814^T (3.1 %). Instead the latter two type strains were distinguished by increased levels of iso-$C_{17:0}$. All four strains shared significant amounts of iso-C_{15} and iso-C_{16} (Table S5). The cell wall of strain HKI0641^T contained *meso*-diaminopimelic acid while mycolic acids were not detected, which is in congruence with the taxonomic position in the genus *Micromonospora*. Whole-cell sugars included arabinose, galactose, glucose, mannose, ribose and xylose. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylinositol mannoside (PIM) were the major polar lipids. Furthermore, trace amounts of phosphatidylglycerol as well as one unknown phospholipid, two glycolipids and three other lipids were found (Fig. S2). The predominant menaquinones were MK-10(H_4) (54 %), MK-10(H_2) (20 %) and MK-10 (13 %), besides small amounts of MK-9(H_4) (4 %), MK-10(H_8) (3 %) and MK-9(H_2) (2 %). The phenotypic properties that differentiate HKI0641^T from the type strains of phylogenetically related species are given in Table 1. In particular, the ability to resort to the glycosides amygdalin or arbutin as sole carbon source appear to be highly distinctive metabolic traits. Furthermore, the type strains *M. aurantiaca* DSM 43813^T and *M. marina* DSM 45555^T, which are phylogenetically most closely related to strain HKI0641^T, can be easily distinguished based upon the colour of their colonies on ISP-4 agar (Table S4).

Resistance genes are widely encountered among antibiotic-producing bacteria to confer self-protection (Cundliffe & Demain, 2010) and, in some cases, the resistance profile of a bacterial strain reflects its potential for the biosynthesis of certain antibiotics (Hotta & Okami, 1996). A set of different antibacterial compounds was hence profiled against the isolated strain HKI0641^T and also against closely related species of the genus *Micromonospora*. Except for novobiocin, all tested antibiotics that are known to be derived from actinomycete bacteria, such as kanamycin, streptomycin, tetracycline, chloramphenicol or vancomycin, were active against the strains of species of the genus *Micromonospora* (Table 1). It appeared thus unlikely that the observed antimicrobial effects of strain HKI0641^T could be ascribed to any of these compounds. To identify the metabolites that account for its biological activity, repeat fermentations were carried out on a 200 l scale in production medium (2 % D-sucrose, 0.2 % casitone, 0.5 % cane molasses, 0.01 % $FeSO_4 . 7H_2O$, 0.02 % $MgSO_4 . 7H_2O$, 0.05 % NaI and 0.5 % $CaCO_3$) at 30 °C. At the end of cultivation, the culture supernatant was separated from the cells by centrifugation at 11 710 ***g*** and extracted with ethyl acetate. The extract was fractionated by open column chromatography on silica gel 60 using a dichloromethane-methanol gradient and, subsequently, on Polygoprep 60-50 C18 (Macherey–Nagel) using a methanol–water gradient. Fractions that showed activity in the agar diffusion assay were pooled and subjected to semipreparative reverse phase-HPLC. After an initial separation on a Nucleodur C18 HTec column (5 µm, VP 250/10, Macherey–Nagel; eluent: 80 % methanol) the final purification of the active component was achieved on a Nucleodur C18 PAH column (3 µm, VP 250/8, Macherey–Nagel; eluent: 80 % acetonitrile). This approach yielded 7.6 mg of the known antibiotic telomycin (Figs S3 and S4), which was identified by comparison of its spectroscopic data with those published in the literature (Kumar & Urry, 1973). Further testing revealed that the observed bioactivity of strain HKI0641^T is largely due to the production of telomycin. The activity profile of the isolated peptide antibiotic was consistent with previous reports (Sheehan *et al.*, 1968). To the best of our knowledge, this is the first report of telomycin production in a species of the genus *Micromonospora*.

Consolidating morphological, biochemical and genetic data, it is evident that strain HKI0641^T exhibits all characteristic features of the genus *Micromonospora*. The strain can be distinguished from the most closely related species of the genus *Micromonospora* by both physiological and genetic traits. The deviations in the 16S rRNA and the *gyrB* gene sequences from those of species of the genus *Micromonospora* with validly published names as well as DNA–DNA hybridization data suggest that HKI0641^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora schwarzwaldensis* sp. nov. is proposed.

Description of *Micromonospora schwarzwaldensis* sp. nov.

Micromonospora schwarzwaldensis (schwarz.wald.en′sis. N.L. fem. adj. *schwarzwaldensis* of or belonging to Schwarzwald, the region where the type strain was isolated).

Gram-stain-positive and strictly aerobic, mesophilic actinomycete. Colonies on ISP-2 agar are orange. Well-developed and branched substrate hyphae bear black, smooth-surfaced spores with a diameter of 600 nm. Aerial hyphae are not produced. Growth is good on ISP-2, ISP-3 and ISP-4 agar and moderate on ISP-5 agar. The growth temperature range is 20–37 °C. Optimal growth occurs at 28 °C. Grows at pH 6–9 and in the presence of <4 % NaCl. Utilizes cellobiose, lactose, maltose and salicin as sole carbon sources for energy, but not fucose, melicitose or raffinose. The diagnostic diamino acid of the cell-wall peptidoglycan is *meso*-diaminopimelic acid. Whole-cell sugars include arabinose, galactose, glucose, mannose, ribose and xylose. The predominant menaquinone is

Table 1. Phenotypic features of strain HKI0641^T and closely related species of the genus *Micromonospora*

Strains: 1, *Micromonospora schwarzwaldensis* sp. nov. HKI0641^T; 2, *M. aurantiaca* DSM 43813^T; 3, *M. coxensis* DSM 45161^T; 4, *M. marina* DSM 45555^T; 5, *M. purpureochromogenes* DSM 43821^T; 6, *M. carbonacea* DSM43168^T; 7, *M. tulbaghiae* DSM 45142^T. All strains were positive for utilization of cellobiose, aesculin, galactose, glucose, glycerol, glycogen, lactose, maltose, sucrose and starch and negative for utilization of *N*-acetylglucosamine, D-adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, gentiobiose, inositol, D-lyxose, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-β-D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, L-rhamnose, D-sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol and L-xylose. All strains showed resistance to penicillin G (10 units), ampicillin + sulbactam (10 + 10 μg), methicillin (5 μg), lincomycin (2 μg) and novobiocin (5 μg), but were sensitive to kanamycin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), chlortetracycline (30 μg), oxytetracycline (30 μg), rifampicin (5 μg), vancomycin (30 μg), azlocillin (75 μg), ciprofloxacin (5 μg), imipenem (10 μg), polymyxin B (300 units), amoxicillin + clavulanic acid (20 + 10 μg), norfloxacin (10 μg). +, positive; W, weakly positive; −, negative.

Characteristic	1	2	3	4	5	6	7
Utilization of:							
Amygdalin	+	−	−	−	W	−	W
L-Arabinose	+	+	+	W	−	−	W
Arbutin	+	−	−	−	W	−	−
Fructose	+	+	+	+	W	−	−
Mannitol	−	+	−	−	W	−	−
Mannose	W	−	W	+	W	−	W
Melibiose	−	+	W	W	+	−	W
Raffinose	−	−	+	−	+	W	W
D-Ribose	W	−	+	W	W	−	−
Salicin	+	+	+	W	W	+	−
Trehalose	W	W	W	−	W	+	−
D-Xylose	+	−	+	+	+	W	W
Temperature for growth (°C)	20–37	25–37	20–37	20–37	20–37	20–37	20–37
pH for growth	6–9	6–10	6–10	6–10	6–10	6–10	7–9
Tolerance to 2 % NaCl	+	+	+	+	+	+	+
Tolerance to 4 % NaCl	+	+	−	−	+	+	+
Resistance to sulphonamide (200 μg)	+	−	+	−	−	+	−

*Results were taken after incubation at 28 °C for 7 days.
†Spores observed after 7 days of cultivation.

MK-10(H_4). The phospholipid profile comprises DPG, PE, PI and PIM. Major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{15:0}$ and $C_{16:0}$.

The type strain is HKI0641^T (=DSM 45708^T=CIP 110415^T), isolated from soil near the Schwarzenbach dam, Germany. The type strain produces the antibiotic telomycin.

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